EJFA Emirates Journal of Food and Agriculture

Emirates Journal of Food and Agriculture 36: 1–15
doi: 10.3897/ejfa.2024.124368

RESEARCH PAPER

Fermentation of soymilk with *Lactobacillus acidophilus* enhances the functional attributes and health-promoting properties

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Academic editor: Angelo Maria Giuffrè ♦ **Received** 2 April 2024 ♦ **Accepted** 27 August 2024 ♦ **Published** 29 October 2024

Abstract

Fermented soymilk is a traditional oriental food beverage that has various health benefits due to its bioactive compounds. However, the fermentation process and the probiotic strains used can affect the quality and functionality of fermented soymilk. In this study, we designed to develop a novel functional food by fermenting soymilk with *Lactobacillus acidophilus* (*L. acidophilus*), a probiotic bacterium that can improve microbiota present in intestine and modulate the immune system. We evaluated the physicochemical, microbiological and sensory attributes of the fermented soymilk, as well as its total phenolic, flavonoids, free amino acids, antioxidant and anticancer activities. The results revealed that *L. acidophilus* had ability to grow well and produce lactic acid in soymilk, resulting in a pH of 4.4 and a titratable acidity of 0.72 g of lactic acid per 100 mL after 12 h of fermentation. The fermented soymilk had a smooth texture, a pleasant aroma and a sour taste, and was well accepted by the panelists. The fermented soymilk also exhibited a good amount of total phenolic, flavonoids, free amino acids with high antioxidant activity towards DPPH free radicals (IC_{50} = 55.51 µg/mL) and the inhibition of human colorectal cancer cells (HCT-116) in a dose-dependent manner (IC_{50} = 67.13 µg/mL). Overall, these findings suggest that fermented soymilk with *L. acidophilus* is a novel functional food with potential therapeutic values for the prevention and treatment of oxidative stress, cancer and metabolic disorders.

Keywords

Fermented soymilk, health benefits, *Lactobacillus acidophilus*, nutraceuticals, probiotic bacteria

Introduction

In the search for innovative and health-promoting food products, A growing field of functional foods has emerged from the intersection of traditional practices and modern science (Cencic and Chingwaru 2010; Damián et al. 2022). Among these, fermented products enriched with probiotics have garnered significant attention for their potential to contribute not only to gastronomic delight but also to human well-being (Soemarie et al. 2021). As consumers increasingly seek dietary choices that not only satisfy their palates but also contribute positively to their overall health, fermented

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foods incorporating probiotics are becoming increasingly popular (Parvez et al. 2006; Rezac et al. 2018; Allaith et al. 2022; Bishop et al. 2022). Probiotics, identified as living organisms that provide health benefits when ingested in proper amounts are linked to better digestive well-being, boosted immunity, and possible protection against different health conditions (Ashraf et al. 2022; Vera-Santander et al. 2023). Harnessing the potential of these microorganisms in tandem with the nutritional richness of soymilk, renowned for its plant-based protein content and other bioactive compounds, presents an exciting avenue for the creation of a functional food that transcends the boundaries of conventional nourishment (Rizzo and Baroni 2018; do Prado et al. 2022).

Fermented soymilk is a traditional oriental food beverage that are produced by fermenting a soymilk containing various bacteria, including lactic acid bacteria (LAB) (Li et al. 2012). Fermented soymilk has several advantages over plain soymilk, like improved taste, digestibility as well as nutritive value (Jayachandran and Xu 2019). Moreover, fermented soymilk can provide unique health advantages to the consumers, such as lowering cholesterol, blood pressure and blood glucose levels, modulating immune system and preventing or alleviating some diseases (do Prado et al. 2022). Among the LAB used for fermenting soymilk, probiotics are of particular interest, as they provide health benefits to the host (Castellone et al. 2021). Probiotics can enhance the bioactivity of fermented soymilk by producing various bioactive substances, such as peptides, vitamin, γ – aminobutyric acid and isoflavone aglycones (Sharma et al. 2022). Furthermore, probiotics can exert antimicrobial activity against pathogenic bacteria and improve the intestinal microbiota balance (Silva et al. 2020).

One of the most popularly utilized and studied probiotic strains is *Lactobacillus acidophilus* (*L. acidophilus*), a homo-fermentative LAB strain (Troche et al. 2020). There has been evidence that it has therapeutic effects on the human body, such as lowering cholesterol, enhancing immunity, preventing diarrhea and inhibiting several bacteria pathogens (Gao et al. 2022). A number of physicochemical properties of soymilk, including its pH, viscosity and nutrient composition, play a crucial role in its nutritional quality and sensory attributes (Olías et al. 2023). Soymilk typically has a neutral to slightly acidic pH and a smooth, creamy texture. These properties can be significantly altered by fermentation (Harper et al. 2022; Statsenko et al. 2023). Fermentation with *L. acidophilus* can boost the nutritional profile of soymilk by enhancing the bioavailability of isoflavones and other nutrients (Kumari et al. 2022; Riasatian et al. 2023). During fermentation, the bacteria produce lactic acid, an acid that decreases the pH of soymilk and can lead to the degradation of anti-nutritional factors, thereby enhancing nutrient absorption (Arbab et al. 2023). The activity of fermentation on the physical parameters of soymilk is multifaceted. The reduction in pH can cause protein coagulation, affecting the smoothness and viscosity of the final product. These changes are important for the sensory characteristics of fermented soymilk, such as taste, texture and aroma, which are critical for consumer acceptance. Additionally, fermentation can produce bioactive peptides with anti-radical and anticancer properties, further enhancing their health benefits (Li et al. 2017; Siddiqui et al. 2023).

Microbiologically, fermentation improves the safety and shelf-life of soymilk through lowering the presence of pathogenic microorganisms and promoting the growth of important bacteria. This not only enhances the health benefits but also makes the product safer for consumption (Kumari et al. 2022; Leeuwendaal et al. 2022; Hasan et al. 2023). This study aims to develop a novel functional food by fermenting soymilk with *L. acidophilus* and to evaluate its physicochemical, microbiological, sensory, antioxidant, and anticancer properties. By elucidating these aspects, we seek to demonstrate the potential of fermented soymilk as a valuable addition to the functional foods industry , offering both enhanced nutritional benefits and improved sensory qualities. Although the development of fermenting soymilk product is not new, this study introduces several innovative aspects that set it apart from previous research. Firstly, it provides a comprehensive analysis of the fermented soymilk, examining not only its physicochemical and microbiological properties but also its sensory, antioxidant and anticancer properties in an integrated manner. Secondly, this study uniquely focuses on the detailed investigation of the anticancer properties of fermented soymilk, offering new insights into its potential therapeutic benefits. Lastly, it incorporates a thorough sensory evaluation by consumers to assess product acceptability, which is crucial for practical applications and marketability. These novel elements collectively contribute to a deeper understanding and greater potential impact of fermented soymilk as a functional food.

Materials and methods

Cultures propagation

The *Lactobacillus acidophilus* MTCC-10307 strain (*L. acidophilus*) was sourced through the Microbial Type Culture Collection (IMTECH, Chandigarh, India). Growth of *L. acidophilus* was conducted on De Man, Rogosa, and Sharpe (MRS) agar plates (Hi-media, Mumbai, India). Meanwhile, bacterial sample stored at 4 °C for subsequent applications.

Screening for probiotic properties

Bile salt tolerance

Tests for bile salt tolerance of *L. acidophilus*, a tube method was used with varying bile salt concentrations. The method involved inoculating 1% of fresh culture of *L. acidophilus* into MRS broth (Hi-media, Mumbai, India) with bile salts (Hi-media, Mumbai, India) ranging from 0.2 to 2%. The samples cultured for 2 days at 37 °C in anaerobic settings, and their growth was assessed using optical density (OD) readings at 560 nm. These readings were juxtaposed with those from a reference tube devoid of bile salts (Vinderola and Reinheimer 2003).

Acid tolerance

Following modifications to the method of Erkkilä and Petäjä (2000), the survival rate of *L. acidophilus* we assessed under extremely acidic conditions. An overnight *L. acidophilus* culture was initiated and transferred to fresh MRS broth (Hi-media, Mumbai, India) until the OD at 600 nm ranged between 0.6 and 0.7. Subsequently, the cell mixture underwent centrifugation at 8000 rpm and 4 °C for 10 min (Eppendorf, Hamburg, Germany). The resultant cell pellet was rinsed and again dissolved in PBS solution at pH - 7.0 (Hi-media, Mumbai, India). From this, 1 mL of solution was combined with 10 mL PBS adjusted to varying pH values of 1, 2, 3, and 7 (control). These mixtures were then incubated anaerobically at 37 °C for 6 h. After incubation, 0.1 mL of the mixture was plated onto MRS agar plates to determine viable cell counts. Furthermore, cell viability was measured after a 24-hour incubation period at 37 °C.

Assessment of cell surface hydrophobicity

By following the protocol demonstrated by Rosenberg et al. (1980), the hydrophobicity of cell surfaces was investigated using xylene, ethyl acetate, and chloroform (Hi-media, Mumbai, India). After being harvested overnight, the *L. acidophilus* culture was twice rinsed with PBS and then reconstituted in 3 mL of PBS buffer. By adjusting the cell density to 10^8 CFU/mL, an initial absorbance ($A_{initial}$) of roughly 0.8–0.9 at 610 nm achieved. 1 mL of each solvent xylene, ethyl acetate, and chloroform was added to each cell suspension separately. Later on, samples were left to incubate for 10 minutes at 37 °C. Furthermore, to separate the organic and aqueous phases, the mixtures were vortexed for 2 minutes and then allowed to settle for 20 minutes at 37 °C. Following the extraction of the aqueous layer, the absorbance at 610 nm (A_{final}) were evaluated. Hydrophobicity of the cell surface was calculated using the formula $[1 - (A_{final} / A_{initial}) \times 100]$.

Ability to aggregate

To evaluate *L. acidophilus* auto- and co-aggregation capabilities, individual cell communication patterns were examined. The auto-aggregation protocol was adapted from Oh et al. (2018). Initially, an overnight culture of *L. acidophilus* was harvested and rinsed twice with PBS (HiMedia, Mumbai, India). This bacterial culture was further reconstituted using 3 mL of PBS, ensuring an initial absorbance ($A_{initial}$) of approximately 0.5 at 600 nm, indicative of a bacterial density of 10^8 CFU/mL. Upon settling, the suspension was kept at 37 °C for 2 h, following which the supernatant absorbance was measured at 600 nm (A_{final}). The auto-aggregation percentage was subsequently derived using following specific calculations;

$$[1 - (A_{final} / A_{initial}) \times 100]$$

To investigate the co-aggregation dynamics among the *L. acidophilus* and other various pathogens (A_{mix}), the method presented by Oh et al. (2018), was followed. Pathogens including *S. marcescens*, *E. coli*, *C. violaceum* and *P. aeruginosa* were suspended in PBS (HiMedia, Mumbai, India) to achieve an absorbance of roughly 0.5 at 600 nm ($A_{pathogen}$), indicating a bacterial concentration of 10^8 CFU/mL. Subsequently, an equivalent volume of each pathogen suspension was combined with *L. acidophilus* (designated as $A_{L.\ acidophilus}$). After a brief 10 sec. vortex (REMI, Mumbai, India), the mixtures were incubated at 37 °C for 5 h. For control purposes, individual bacterial suspensions (2 mL each) were mixed without the addition of *L. acidophilus*. Post-incubation, a spectrophotometer was used to measure the absorbance of the resulting solutions at 600 nm (Shimadzu Corporation, Kyoto, Japan). Co-aggregation percentages were then computed using a following specific formula.

$$[(A_{L\ actobacillus} + A_{pathogen})/2 - (A_{mix})/(A_{L.acidophilus} + A_{pathogen})/2] \times 100$$

Hemolytic activity

Following the method outlined by Gunyakti and Asan-Ozusaglam (2019), with certain modifications, the hemolytic potential of *L. acidophilus* was examined. An overnight culture of *L. acidophilus* was streaked onto an MRS blood agar plate (Hi-media, Mumbai, India), followed by incubation for 48 h at 37 °C. Subsequent assessment of the culture involved examining the hemolysis zones adjacent to the bacterial colonies. The appearance of a clear zone indicated beta hemolysis, a greenish shade was indicative of alpha hemolysis, while the absence of any discernible zone denoted gamma hemolysis.

Soymilk preparation and fermentation

Following the method proposed by Tang et al. (2018), soymilk was produced and fermented using *L. acidophilus* as mentioned in Fig. 1. Soybeans locally procured were stored at 4 °C until required. These soybeans underwent a thorough washing before being soaked overnight in water, equivalent to twice their weight. After soaking, the soybeans were combined with water, amounting to four times their weight, and blended for a duration of 3 min. Soymilk was extracted from the resulting mixture using a double-layered cloth. This soymilk was then transferred to sterilized bottles and subjected to autoclaving at 105 °C for 15 min. Once cooled, *L. acidophilus* was introduced to the soymilk at a concentration of 2% (v/v), after which it was incubated at 37 °C for a period of 12 h.

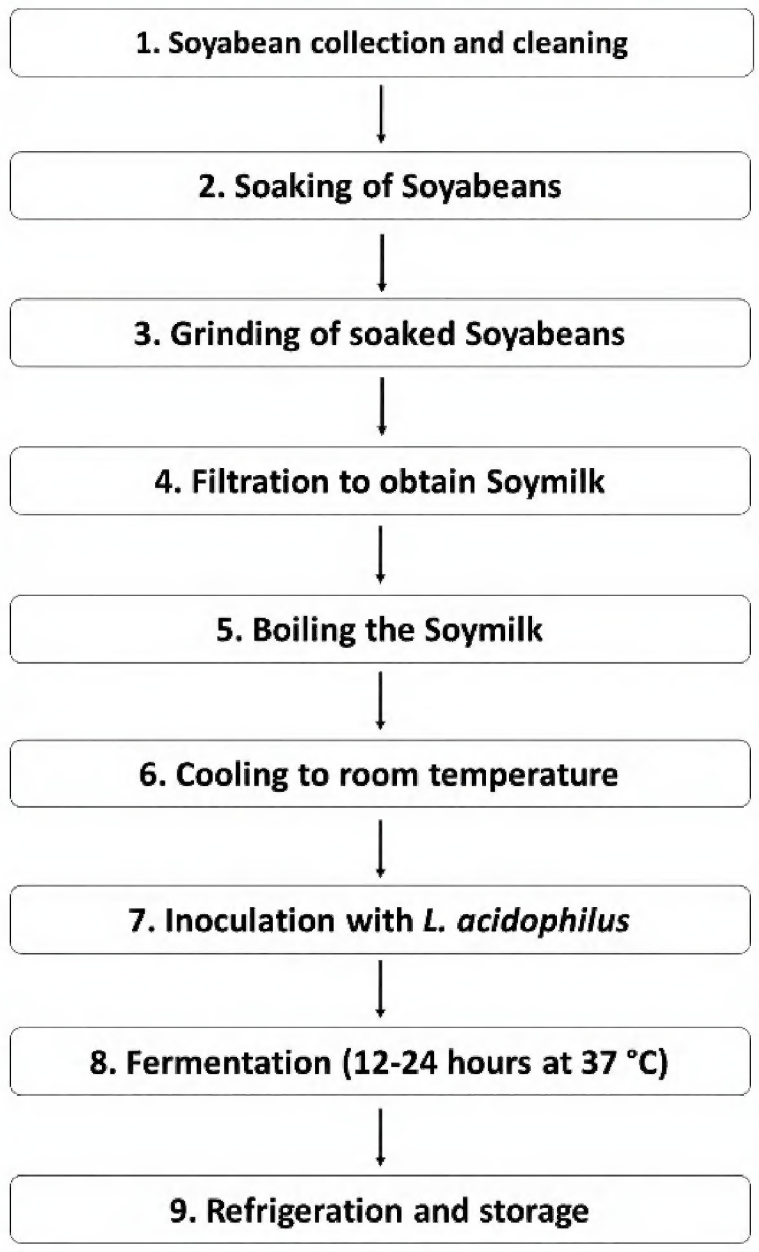
1. Soyabean collection and cleaning
2. Soaking of Soyabeans
3. Grinding of soaked Soyabeans
4. Filtration to obtain Soymilk
5. Boiling the Soymilk
6. Cooling to room temperature
7. Inoculation with L. acidophilus
8. Fermentation (12-24 hours at 37 °C)
9. Refrigeration and storage

Figure 1. Various steps involved in the development of novel soymilk fermented food.

Determination of probiotic viability

The probiotic strain, *L. acidophilus*, was quantified through standard plate count assay. Using a 0.9% NaCl solution (Hi-media, Mumbai, India), serial dilutions of fermented beverages were prepared. From these dilutions, 1 mL aliquots were spread over MRS agar plates. These plates underwent incubation for 48 h at 37 °C in anaerobic settings. For enumeration, plates containing 25 to 250 colonies were chosen, and the results were reported in CFU/mL. All tests were conducted in triplicate (Maftei and Iancu 2023).

Determination of pH and acidity

A pH meter (Mettler Toledo, Greifensee, Switzerland) was used to determine the sample's pH. 10 mL of the centrifuged beverage supernatant were titrated with 0.1 N NaOH (Hi-media, Mumbai, India) in order to determine the titratable acidity. The indicator used for this titration was phenolphthalein (Hi-media, Mumbai, India), which becomes pink at pH 8.3. The sample was first given two to three drops of phenolphthalein. Then, while stirring continuously, 0.1 N NaOH was gradually added to the sample to titrate it. When a steady, faint pink tint emerged, signifying neutrality, the titration deemed to be completed. Grams of lactic acid per 100 milliliters of the fermented solution is considered as the unit of measurement for titratable acidity (Baú et al. 2014).

Water Holding Capacity (WHC)

In this study, Pyo and Song (2009), method was employed, with certain adjustments, for calculating water holding capacity (WHC). Essentially, 10 g of the beverage was transferred to a 20 mL centrifuge tube and subjected to centrifugation at 2500 rpm for 10 min at 20 °C (Eppendorf, Hamburg, Germany). As soon as the water was expelled, and its weight recorded. The WHC percentage was subsequently computed using a specific formula.

$$\text{WHC} = [(\text{weight of sample} \times \text{expelled water}) / \text{weight of sample}] \times 100$$

Preparation of solvent extracts

By following the procedure of Tang et al. (2018), the extraction process was initiated. Whether the soymilk was fermented or non-fermented, it underwent freeze-drying using a freeze dryer (Atlas Copco, Nacka, Sweden). Subsequently, the freeze-dried product was subjected to ultrasound sonicator (Analytical Technologies Limited, Vadodara, India) (at 100 W) in the presence of a solvent, which could either be water or 80% ethanol, with a ratio of 1:10 (w/v). The mixture was kept at 25 °C for 6 h. Following this, the extracts were centrifuged at 10,000 rpm for 10 min at 4 °C (Eppendorf, Hamburg, Germany). The resultant supernatants were harvested and subjected to a second round of freeze-drying. These freeze-dried extracts were then preserved at a temperature of −80 °C.

Determination of total phenolic content

By following the procedure given by Chen et al. (2013), the total phenolic content of the prepared samples was assessed. To begin, the freeze-dried samples were reconstituted using 0.1 mL of DMSO (HiMedia, Mumbai, India). This solution was combined with 1.9 mL of D/W, followed by the addition of 1.0 mL of Folin–Ciocalteu phenol reagent (HiMedia, Mumbai, India) in a 1:10 proportion. 5.0 mL of a 20% Na_2CO_3 solution (HiMedia, Mumbai, India) was added to this mixture, and the reaction was allowed to continue for 20 minutes at room temperature without light. Following incubation, the samples' absorbance at 735 nm was measured. The results were represented as milligrams of gallic acid per milligram of the extract and the total phenolic content was calculated by comparing the results to a standard curve made with gallic acid (Hi-media, Mumbai, India).

The free amino acid content measurement

Following the methodology outlined by Xing et al. (2017), the free amino acid in both fermented and non-fermented soymilk were assessed. The initial step involved adjusting the pH of the soymilk samples, both fermented and

unfermented, to a value of 4.6. These samples were subsequently subjected to centrifugation at 3,000 rpm for 30 min at 4 °C to separate the supernatants. To 50 µL of these supernatants, 2 mL of O-phthalaldehyde (Hi-media, Mumbai, India) was added, followed by a 2 min incubation period at 37 °C. The optical density of the resultant solution was then recorded at 340 nm (Shimadzu Corporation, Kyoto, Japan). Utilizing a standard curve derived from a Leucine standard (Hi-media, Mumbai, India), the free amino acid concentration was determined with outcomes presented as milligrams of Leucine present in the extract.

Determination of flavonoids

Utilizing the aluminum chloride method described by Shwetha et al. (2011), with rutin (Hi-media, Mumbai, India) as standard, the quantity of flavonoids present in each sample was determined. An aliquot comprising 1 mL of the sample extract was combined with 4 mL of water in a volumetric flask. Following this, 0.3 mL portions of both 5% sodium nitrite (Hi-media, Mumbai, India) and 10% aluminum chloride (Hi-media, Mumbai, India) solutions were added. The mixture was then kept to equilibrate at 37 °C for 5 min. To this solution, 2 mL of a 1M sodium hydroxide solution (Hi-media, Mumbai, India) was incorporated, and adjusted to 10 mL using D/W. With a spectrophotometer (Shimadzu Corporation, Kyoto, Japan), the absorbance of the prepared solution was measured at 510 nm and a blank as reference were employed. Furthermore, by using a standard curve constructed from rutin, the flavonoid concentration in the sample was determined. The results were expressed as milligrams of flavonoids per milligram of extract.

Sensory analysis

Faculty members from the Food Technology Department of Parul University, India, comprising 2 males and 4 females with age group of 24–45, possessing partial training in sensory evaluation, were tasked with evaluating the sensory attributes of the fermented soymilk samples. After being removed from refrigeration, these samples underwent assessment using a 9-point Hedonic scale, which encompassed parameters such as color and appearance, body and texture, acidity, flavor, and overall acceptability. This scale spanned from a score of 1, indicating strong disapproval, to 9, representing strong approval. The adoption of the 9-point Hedonic scale for this evaluation was based on its recognized utility in measuring product acceptance and preference (Raju and Pal 2014).

Determination of antioxidant potential

The procedure of Brand-Williams et al. (1995), was modified to measure the DPPH$^{\bullet}$ scavenging activity of fermented soymilk product. Working solutions with various concentrations (5–100 µg/mL) were prepared from fermented soymilk product (100 µg/mL). A 0.2 mM DPPH$^{\bullet}$ solution (Hi-media, Mumbai, India) in methanol was also formulated. Each working solution of fermented soymilk product (100 µL) was mixed with an equal amount of DPPH$^{\bullet}$ solution. The Incubation of the reaction mixture was carried out at 37 °C in a dark condition for 30 min. The reduction of DPPH$^{\bullet}$ was indicated by the colour change from violet to yellow. The absorbance of samples was measured at 517 nm by ELISA plate reader (BioTek, USA). Ascorbic acid (Hi-media, Mumbai, India) was the reference and DPPH$^{\bullet}$ was the control. The formula below was used to calculate the DPPH$^{\bullet}$ scavenging percentage of fermented soymilk product:

$$\text{DPPH scavenging activity} = (\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) / (\text{Abs}_{\text{control}}) \times 100$$

Determination of cytotoxicity

The cytotoxic impact of fermented soymilk product on HCT-116 cell lines was assessed using the MTT assay. HCT-116 cells were introduced into 96-well tissue culture plates, with each well containing 1.5×10^4 cells and allowed to adhere over 24 hrs. Subsequently, the cells were exposed to varied concentrations (ranging from 5–100 µg/mL) of the fermented soymilk product and were further cultured for 24 h at 37 °C in an environment with 5% CO_2. Following this, 100 µL of MTT solution (5 mg/mL) (Hi-media, Mumbai, India) was introduced to each well and incubated for an additional 4 h under the same conditions to yield a purple precipitate. The content of the wells was then aspirated, and 150 µL of DMSO (Hi-media, Mumbai, India) was used to dissolve the purple residue. Using an ELISA plate reader (BioTek, USA), the absorbance of the samples was taken at 570 nm. This entire procedure was carried out thrice. The IC_{50} value, representing the concentration where 50% of cell viability impeded was derived. The formula to compute cell viability (%) is indicated below (Elasbali et al. 2022).

$$\%\ \text{Cell viability} = \text{Absorbance at 570 nm of treated cells} / \text{Absorbance at 570 nm of control cells} \times 100$$

Statistical analysis

The experiments were replicated three times, and the mean ± standard deviation (SD) of the repeated trials is used to represent the results. After determining the statistical significance using a one-way ANOVA, a multiple comparisons test using Dunnet's was performed with a significance level of $p < 0.05$.GraphPad Prism 8.0 was used for all analyses.

Results and discussion

Fermented soymilk with probiotic LAB has emerged as an innovative functional food with potential health benefits (Soemarie et al. 2021). The current approach represents

a comprehensive study on the development and evaluation of fermented soymilk with probiotic *L. acidophilus*. Soymilk, a widely consumed plant-based beverage, is an ideal medium for probiotic bacteria due to its nutritional profile and mild flavor profile (Li et al. 2012; Jayachandran and Xu 2019). The successful colonization of the probiotic bacteria in the fermented soymilk highlights the potential of this formulation as a probiotic supplement. Probiotics have shown a range of health advantages, such as enhanced digestive functions, increased immune response and diminished susceptibility to certain ailments (Das et al. 2022; Ashraf et al. 2022; Vera-Santander et al. 2023). The probiotic *L. acidophilus* strain used in this research possesses specific probiotic properties, which further enhance its efficacy as a functional food. The favorable taste and texture of the fermented soymilk are key factors for its acceptance and consumption. By incorporating probiotic bacteria into soymilk, it is possible to provide individuals with a convenient and enjoyable way to consume probiotics (Hasan et al. 2023). This fermented soymilk can work as a versatile and functional food ingredient that can be combined into various food products, such as yogurts, smoothies, and beverages, to cater to diverse consumer preferences (Almghawesh et al. 2022; Kumari et al. 2022).

Bile salt and acid tolerance

The resistance of *L. acidophilus* to bile salts was assessed across concentrations ranging from 0.2% to 2%. Findings revealed that *L. acidophilus* remained viable within the 0.2% to 1.2% bile salt range. The bile salt tolerance of *L. acidophilus* decreased progressively after 1.2% of bile salt concentration. To validate the bile salt tolerance, *L. acidophilus* was also examined for its survival under highly acidic conditions. *L. acidophilus* was exposed to pH 1.0, 2.0, 3.0 and 7.0 (control) for 24 h. The results of the pH test indicated that *L. acidophilus* showed no viability at pH 1 after 24 h of incubation. However, it exhibited a good viability at pH 2, 3, 4 and 7 (Table 1). One of the key features for selecting good probiotic candidates is their ability to cope with acid and bile conditions. Probiotics must thrive, proliferate and execute their roles amidst highly acidic environments and elevated bile salt concentrations, representing initial obstacles in the rigorous gastrointestinal environment (Kumari et al. 2021). A majority of *in-vitro* assessments are intended to recognize strains capable of withstanding pH levels between 2.0–3.0 and bile concentrations ranging from 0.3% to 1% (Bhushan et al. 2017; Gunyakti and Asan-Ozusaglam 2019). Consequently, these pH and bile benchmarks were adopted as benchmarks for pinpointing potential probiotic candidates.

The acid tolerance of *L. acidophilus* was demonstrated due to higher number of viable cells at pH 3 compared to pH 2. This finding aligns with the findings of Gunyakti and Asan-Ozusaglam (2019), wherein a greater number of viable cells were observed at pH 3 compared to pH 2. Prior research has also indicated diverse survival rates

Table 1. Bile salt and acid tolerance of *L. acidophilus* after 24 h of incubation.

Bile salt concentration	Results
0.2	+
0.4	+
0.6	+
0.8	+
1	+
1.2	+
1.4	-
1.6	-
1.8	-
2	-
pH	
1	-
2	+
3	+
4	+
7	+

(+ = positive, - = negative).

for *Lactobacillus* strains under acidic conditions (Bhushan et al. 2017; Bhushan et al. 2021). The existence of fermentable sugars can potentially boost the persistence of certain probiotic lactobacilli in acidic settings. This is attributed to their provision of ATP to F0F1-ATPase via glycolysis, facilitating the removal of protons (Corcoran et al. 2005). *L. acidophilus* also showed bile salt tolerance and could grow at both 0.3% and 1.2% bile salt. This data suggests that *L. acidophilus* may have better adaptation to the small intestine conditions. The present results are in consistent with previous research showing significant variation in bile tolerance between *Lactobacillus* strains (Bhushan et al. 2017; Oh et al. 2018). The bile resistance and acid characteristics observed in *Lactobacillus* strains suggest that these traits are unique to individual strains.

Cell surface hydrophobicity

The adhesion ability or property of LAB to the organic compounds is measured by bacterial adhesion to hydrocarbons. The more nonpolar the surfaces are, the more they adhere to hydrocarbons and the more hydrophobic. Fig. 2 shows the percent adhesion of *L. acidophilus* to different hydrocarbons. Chloroform (38.88%) had the highest adhesion score among the three hydrocarbons tested, followed by ethyl acetate (34.33%) and xylene (30.55%). Meanwhile, how effectively a probiotic strain adheres to the gut epithelial cells of the host is indicated by the hydrophobicity of its cell surfaces. One can categorize xylene, ethyl acetate, and chloroform as non-polar solvents, solvents that donate mono-polar electrons, and solvents that take up mono-polar electrons, in that order (Klopper et al. 2018). Differences in the cell surface hydrophobicity would be due to the presence of difference in composition of surface polymers mainly play an important role in hydrophobic association, which might affect the strength of

adhesion (Colloca et al. 2000). The differing propensities of *L. acidophilus* to bond with chloroform (an acidic polar solvent) and ethyl acetate (a basic polar solvent) hint at its potential to reside in mucus (Klopper et al. 2018). Thus, assessing cell surface hydrophobicity can guide the selection of probiotics with superior binding capabilities to the gut epithelial lining.

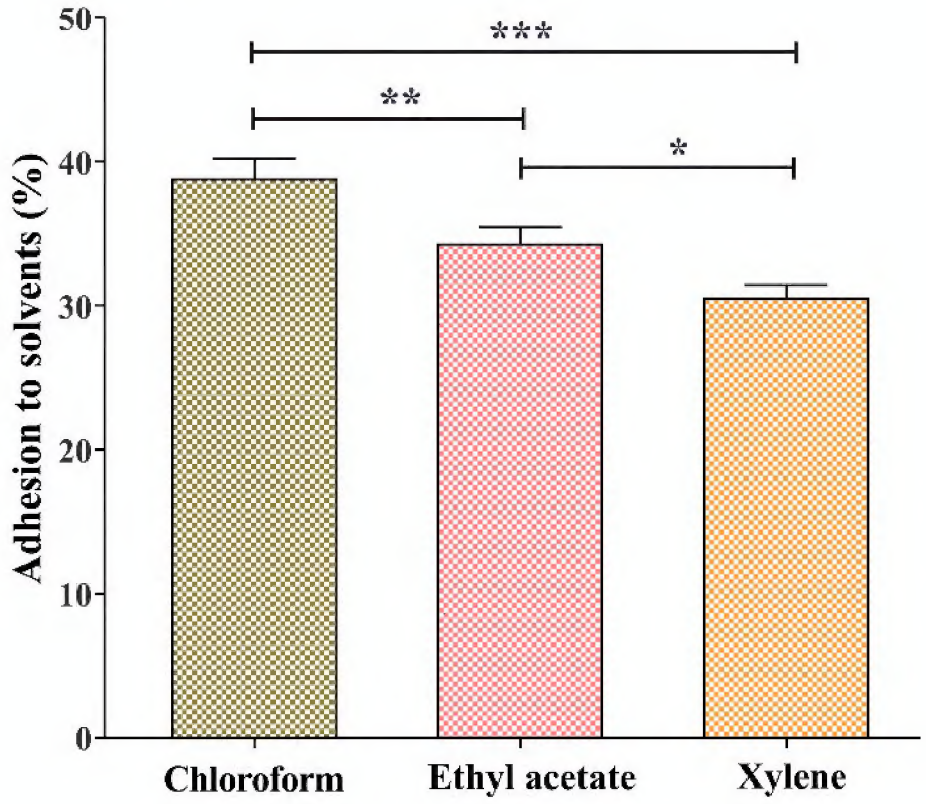

Figure 2. Impact of various solvents (xylene, ethyl acetate and chloroform) on the cell surface hydrophobicity of *L. acidophilus*. Error bars represent the standard deviation (SD) from three independent experiments. Statistical significance; ns > 0.05,* p < 0.05, ** p < 0.005, *** p < 0.0005, compared to each other.

Auto-aggregation and co-aggregation activity

The activity of auto-aggregation for *L. acidophilus* was measured by incubating the bacterial suspension for 3 and 5 h at 37 °C. The percentage of auto-aggregation was determined by matching the absorbance of the initial and final suspensions. The obtained results indicated that *L. acidophilus* had a high auto-aggregating phenotype, with 63.73% and 67.49% activity after 3 and 5 h, respectively (Fig. 3). This indicates that *L. acidophilus* can form stable multi-cellular aggregates that may enhance its colonization and adhesion into the gastrointestinal tract. The co-aggregation activity of *L. acidophilus* was measured by mixing the bacterial suspension with different pathogenic strains, such as *S. marcescens*, *E. coli*, *C. violaceum* and *P. aeruginosa* for 24 h on 37 °C. Furthermore, the % co-aggregation was determined via comparing the optical density of the initial and final suspensions. The results showed that *L. acidophilus* had a highest value for the co-aggregation ability with *E. coli* (28.38%), followed by *C. violaceum* (19.10%), *S. marcescens* (15.00%) and *P. aeruginosa* (11.06%) (Fig. 4). This indicates that *L. acidophilus* can interact with pathogenic bacteria and may inhibit their adhesion and colonization in the gastrointestinal tract. By forming large clusters of probiotic bacteria, the growth of harmful microorganisms can be inhibited and the attachment to the host epithelial cells can be enhanced through competitive exclusion (Krausova et al. 2019). The formation of large clusters depends on the aggregation ability of the probiotic bacteria. *L. acidophilus* had a high aggregation ability. This indicated a strong relationship between aggregation and the hydrophobicity of the microbial cell surface of *L. acidophilus*. This relationship was also observed in previous studies (Klopper et al. 2018). The aggregation of *Lactobacillus* was consistent with previous findings that the binding ability of lactobacilli was specific to each strain (Bhushan et al. 2021). It was previously believed that proteins on the surface of the cells and proteins or lipoproteins present in the culture supernatant had an impact on the clustering of lactobacilli (Klopper et al. 2018). Probiotics ability to co-aggregate with pathogens leads to the expulsion of these pathogens from the intestine through displacement. This is how probiotics exert their therapeutic effect in the disease or infection situation. In this study, we found that *L. acidophilus* had the highest co-aggregation capacity with *E. coli* (28.38%), followed by *C. violaceum* (19.10%), *S. marcescens* (15.00%) and *P. aeruginosa* (11.06%). In our current research, the co-aggregation results for *L. acidophilus* align with the findings of Bhushan et al. (2021), who observed diverse co-aggregation levels of the *Lactobacillus* strain with pathogens such as *S. typhimurium* and *E. coli*. The different levels of co-aggregation and adhesion to the gut epithelium among *Lactobacillus* species indicate that co-aggregation capacity is dependent on the strain (Bhushan et al. 2021). *Lactobacillus* species' capacity for co-aggregation is associated with particular surface chemicals that function as pathogen attachment receptors when they bind to the intestinal epithelium (Oh et al. 2018).

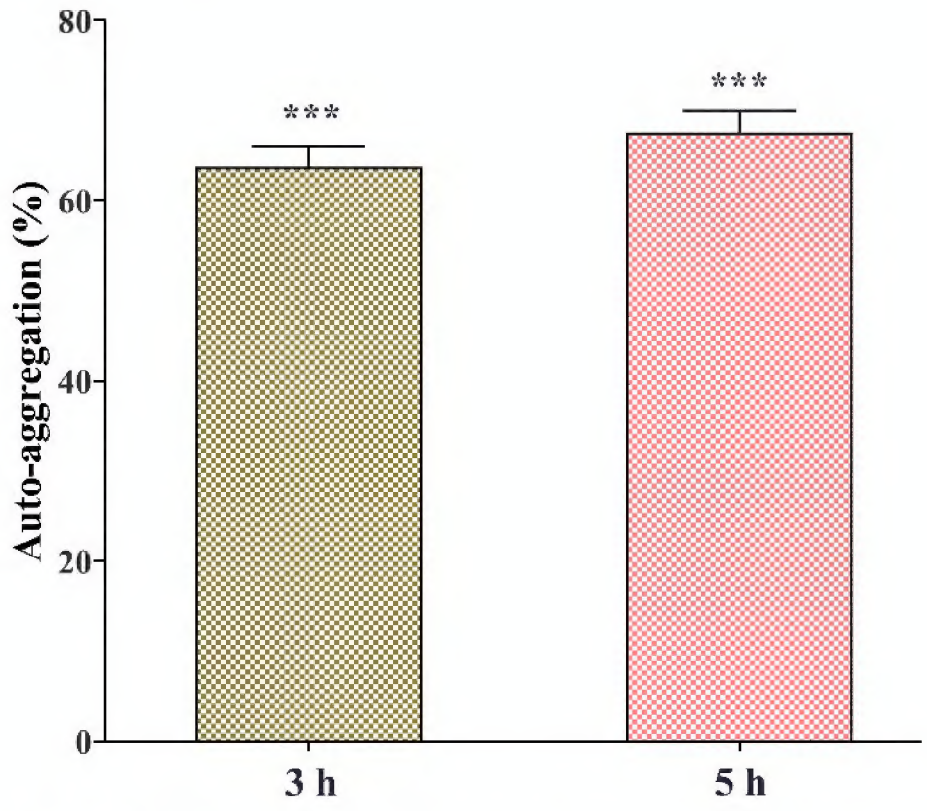

Figure 3. Assessment of *L. acidophilus* auto-aggregation percentage via spectrophotometry. Error bars represent the standard deviation (SD) from three independent experiments. Statistical significance; ns > 0.05,* p < 0.05, ** p < 0.005, *** p < 0.0005, compared to each other.

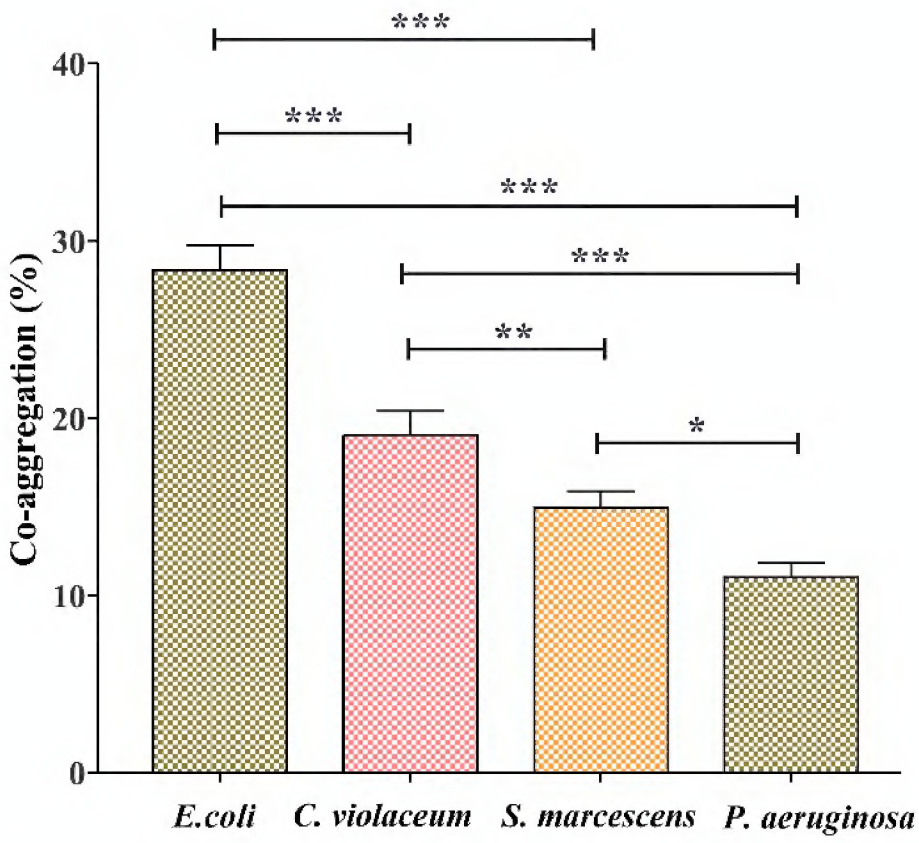

Figure 4. Assessment of *L. acidophilus* co-aggregation percentage via spectrophotometry. Error bars represent the standard deviation (SD) from three independent experiments. Statistical significance; ns > 0.05,* p < 0.05, ** p < 0.005, *** p < 0.0005, compared to each other.

Hemolytic assessment

No distinct transparent or greenish zones were observed around the colonies of *L. acidophilus* on blood agar plates, indicating a γ-hemolytic or non-hemolytic nature (Fig. 5). Before approving a probiotic for its positive impacts on health or application in the food sector, one of the most important characteristics to evaluate is its safety. The main experimental test that is done in this context is hemolysis activity (Casarotti et al. 2017). Hemolysis means the breaking of red blood cells; therefore, probiotic strains that do not have haemolytic activity are preferred (Peres et al. 2014). This examination holds importance since *Lactobacillus* is typically viewed as harmless residents and the absence of hemolysis activity eliminates the chance of opportunistic virulence in the samples (Peres et al. 2014). In this study, *L. acidophilus* did not exhibit any kind of haemolysis. This result aligns with earlier reports wherein various *Lactobacillus* strains displayed no hemolysis (Bhushan et al. 2017). Hence, they might be considered as promising functional organisms.

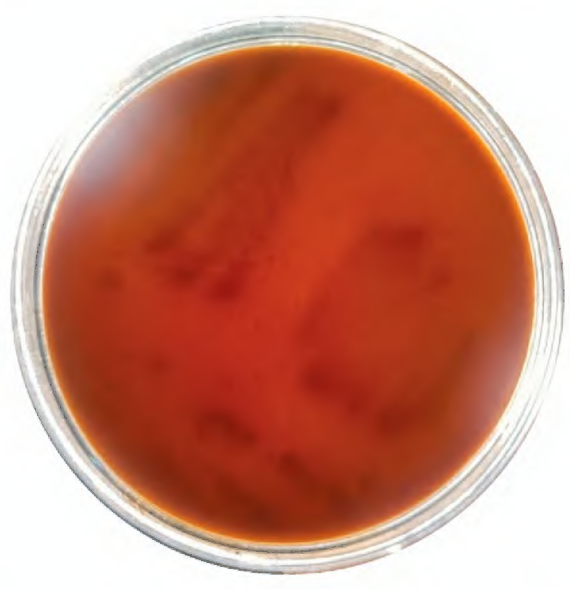

Figure 5. Hemolytic assessment of *L. acidophilus* on blood agar plates showing no distinct transparent or greenish zones, indicating non-hemolytic nature.

Development of soymilk fermentation product

After the successful evaluation of probiotic potential of *L. acidophilus* as a probiotic, it was utilized for the development and preparation of fermented soymilk product. In this novel product (fermented soymilk), assessments were conducted for probiotic viability, pH, acidity, water retention ability, as well as concentrations of total phenolics, free amino acids, and flavonoids (Fig. 6). The product was also tested for its antioxidant activity and its capacity to suppress the human colon cancer cells proliferation. Designing the food matrix for the effective delivery of probiotics is a critical aspect in developing non-dairy probiotic products, as it directly impact the efficiency of probiotics. The health advantages derived from probiotics are not solely reliant on the strain type rather the matrix environment also plays a crucial role. Therefore, selecting a probiotic carrier in the form of a food matrix is paramount to ensure the prolonged viability of the probiotics. As a result, we investigated a number of *in-vitro* variables to assess how the food matrix affected *L. acidophilus*.

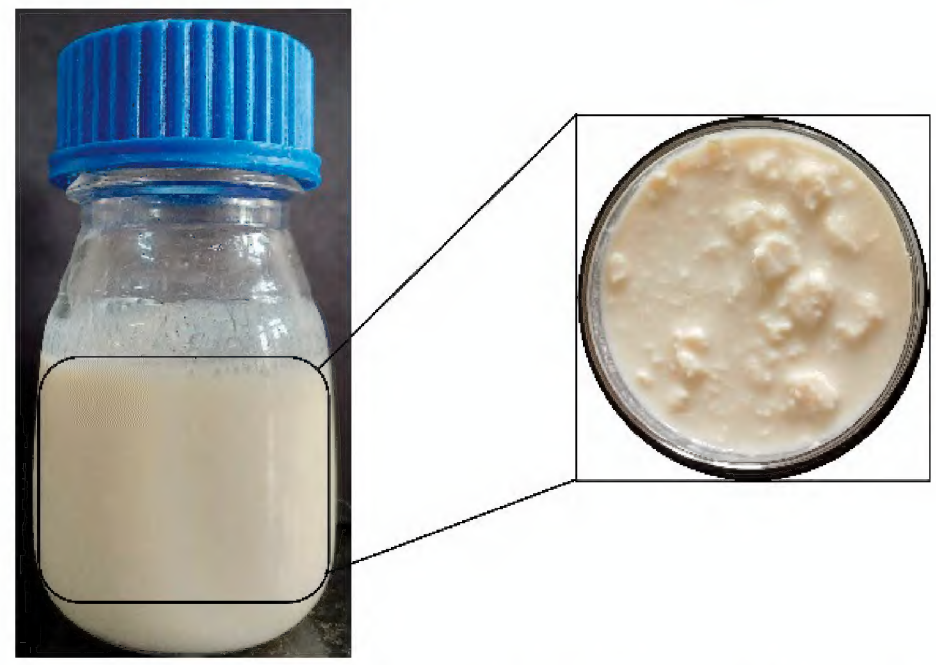

Figure 6. Probiotic *L. acidophilus* fermented soymilk product formulation.

Cell viability and physicochemical changes during fermentation

The viability of *L. acidophilus* in the fermented soy product was assessed at both the onset (time 0) and after (12 h) of the fermentation cycle using the plating technique. Initially, the *L. acidophilus* viability was at 4.7×10^6 CFU/mL. By the initiation of the fermentation, this count had escalated to approximately 2.3×10^8 CFU/mL. Concurrently, the pH had diminished to 4.4 by the end, indicating successful fermentation and the onset of organic acid production by *L. acidophilus*. Additionally, the fermented product exhibited a titratable acidity of 0.72 g of lactic acid per 100 mL (Table 2). The probiotic strain *L. acidophilus* demonstrated

improved viability when exposed to diverse components, including sugars and proteins, in the beverage, supplying essential nutrients. Despite a slight decline in the cell numbers of *L. acidophilus* over the storage period, they consistently remained at approximately 1.8×10^8 CFU/mL. According to Inoguchi et al. (2012), soymilk is known to contain soybean oligosaccharides that lactic acid bacteria can utilize. Furthermore, Korbekandi et al. (2011), highlighted that the fermentation temperature has a substantial impact on the viability of probiotic microorganisms and a range of quality attributes of probiotic fermented products, with the range of 37 to 43 °C being ideal for the growth of most probiotic strains. In order to ensure a favorable interaction that improves microbial viability during both fermentation and storage, it is crucial to investigate the compatibility among probiotics, fermentation conditions, and the food matrix.

Regarding the viable microorganism count at the time of consumption, there is a general consensus in the food industry that a minimum of 10^6 CFU/mL is recommended (Boylston et al., 2004), a suggestion also endorsed by the US FDA. According to Knorr (1998), the body needs to consume 10^8–10^9 probiotics per day to experience the benefits of probiotics, depending on the quantity consumed and how storage affects viability. The live *L. acidophilus* cells in our investigation maintained the required level, proving that our drink meets the criteria for being a functional drink. Furthermore, this implies that soy milk-based beverages could stimulate further research into the development of new, balanced matrices for probiotics.

Following 12 h of fermentation, there was a decline in pH values from the initial reading, accompanied by an increase in cell count throughout the fermentation process. The pH dropped to around 4.4 due to the ongoing metabolic activities of *L. acidophilus* at the end of fermentation. This observation aligns with findings by Yu et al. (2015), who noted that a *Prunus mume* puree (probiotic) exhibited a pH of 3.0, down from an initial 3.5, after a 10-day room temperature storage period. Additionally, a negligible pH shift, from 5.5 to 5.7, was observed over a four-week span in a probiotic cheese-like product (Cichosz et al. 2014). The decline in pH during fermentation can be attributed to organic acid production. A notably low pH amplifies the bactericidal properties of these acids by elevating the percentage of organic acids in fermented items (Tripathi and Giri 2014). Tripathi and Giri (2014), suggested that beverages, particularly those with low pH levels like fruit juices, present a notable hurdle for probiotics. *Lactobacillus* thrives best within a pH range of 6.0–7.0. Further, as highlighted by Maia et al. (2023), *Bifidobacteria* species exhibit lower acid resistance, with pH levels below 4.6 being detrimental to their viability.

Table 2. pH, cell viability, acidity, water holding capacity and Sensory analysis of the soymilk fermented with *L. acidophilus*.

Parameters	Before fermentation (0 h)	After fermentation (12 h)	After storage (21 days)
pH	6.3 ± 0.1	4.4 ± 0.2	3.9 ± 0.2
Cell viability (CFU/mL)	$4.7 \pm 0.3 \times 10^6$	$2.3 \pm 0.3 \times 10^8$	$1.8 \pm 0.26 \times 10^8$
Titratable acidity	0.13 ± 0.05	0.72 ± 0.06	1.16 ± 0.07
Water holding capacity (%)	-	76 ± 2.64	71 ± 1.0
Appearance	-	7 ± 0.57	6.5 ± 0.76
Flavour	-	5.16 ± 0.37	4.83 ± 0.68
Smell	-	4.83 ± 0.37	5.33 ± 0.74
Texture	-	4.5 ± 0.5	4.0 ± 0.57
Overall Acceptance	-	5.37 ± 0.27	5.16 ± 0.53

Changes in cell viability, pH, water holding capacity and titratable acidity during storage

After 21 days of cold storage at 4 °C, the cell viability of *L. acidophilus* moderately reduced to 1.8×10^8 CFU/mL at the end of storage period at 4 °C. The pH values of the fermented soy drinks also reduced during storage. The minimum pH value reached was 3.9. The WHC was 71% during the storage period. On the other hand, the acidity values increased after 21 days of storage time. Meanwhile, the acidity value was about 1.16 g of lactic acid 100 mL^{-1} (Table 2).

As the fermentation progressed for all functional beverages, a reduction in pH was observed, accompanied by a corresponding increase in titratable acidity. Several studies have also shown the increase in titratable acidity (Gautam and Sharma 2014; Obadina et al. 2013). In this study, the post-acidification due to the continued fermentation by *L. acidophilus* during storage can explain the increased acidity of the formulations. In the present study, the recorded titratable acidity and pH values function as indirect markers of starter metabolic activity over the stowing duration. These findings align with our observations regarding *L. acidophilus* viability and the organic acid concentrations over the storage duration. Sheehan et al. (2007) highlighted notable variations in the acid tolerance between *Lactobacillus* and *Bifidobacterium* when introduced to various juices. Specifically, these strains exhibited superior survivability in two juices such as pineapple and orange compared to cranberry juice. Initial WHC measurements surpassed those recorded during the storage phase, with the storage duration having negligible effects on the WHC of the functional beverages. Despite an increase in acidity over time, the functional beverage exhibited minimal water release tendencies. While our findings resonate with those presented by (Aparicio-García et al. 2021), they contrast with conclusions drawn from studies on fermented dairy beverages by other researchers (Lin et al. 2004).

Total phenolic compounds, flavonoids, and free amino acids in fermented soymilk

As the fermentation duration extended, soymilk inoculated with *L. acidophilus* displayed an augmentation in its total phenolic, flavonoid and free amino acid concentrations. The total phenolic contents vary from 12.21 ± 0.97 to 22.99 ± 0.32 mg GAE/g of extract (Fig. 7), whereas the flavo-

noid levels increased from 17.37 ± 1.39 to 22.20 ± 1.46 mg RTE/g of extract (Fig. 8). Concurrently, the content of free amino acids in the extract also increased from an initial 3.5 ± 0.2 mg/g to 10.54 ± 0.4 mg/g (Fig. 9). The soymilk subjected to fermentation by *L. acidophilus* exhibited notable amount of total phenolic compounds, flavonoids and free amino acids. This enhancement is likely attributed to the heightened β-glucosidase activity present in the *L. acidophilus*-fermented soymilk. Elevated amino nitrogen levels hinted at increased protein breakdown and augmented concentrations of amino acids and peptides within the sample. Tang et al. (2018), documented a notable rise in the β-glucosidase activities within *L. acidophilus*-fermented soymilk over a fermentation span of 6 to 24 h. The augmentation in total phenolic content and aglycone isoflavones might be attributed to the phenolics released by the β-glucosidase of *L. casei* 16 throughout the fermentation process. According to Rekha and Vijayalakshmi (2011), certain *Lactobacilli* strains possess β-glucosidase enzymes capable of converting soymilk's isoflavone glucosides into aglycone isoflavones. Moreover, the β-glucosidase enzymes sourced from lactic acid bacteria, as emphasized by Donkor et al. (2010), possess the ability to catalyze the cleavage of the β-1-6 glucosidic bond that links the isoflavone pran ring and sugar moieties. Through the fermentation process, this enzymatic activity can transform isoflavone glycosides into sugar components and bioactive isoflavone aglycones, which can improve the biological efficacy of soymilk. According to Chen et al. (2013), black soybeans fermented with *Aspergillus awamori* showed an increase in their peptide and amino acid profiles. According to Su et al. (2018), the solid-state fermentation of soybeans led to a better breakdown of soybean proteins due to a mix of *Lactobacillus* species and *Clostridium butyricum*.

Sensory acceptability

The sensory parameters of the soymilk fermented with *L. acidophilus* was determined using a 9-point hedonic rating system, with values ranging from 1 (strongly dislike) to 9 (strongly like). Attributes such as appearance, color, aroma, taste, texture, and overall liking were evaluated. The results are shown in Table 2.

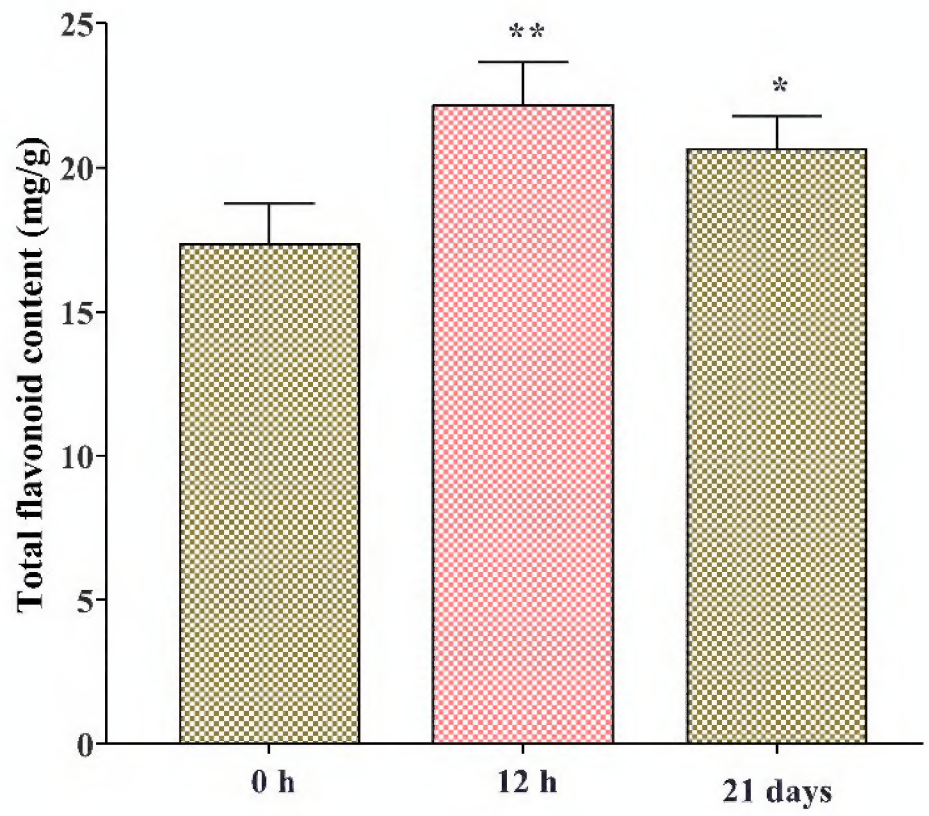

Figure 8. Quantification of flavonoids in soymilk pre- (0 h) and post- (12 h and 21 days) *L. acidophilus* fermentation at 37 °C. Error bars represent the standard deviation (SD) from three independent experiments. Statistical significance; ns > 0.05,* p < 0.05, ** p < 0.005, *** p < 0.0005, compared to each other.

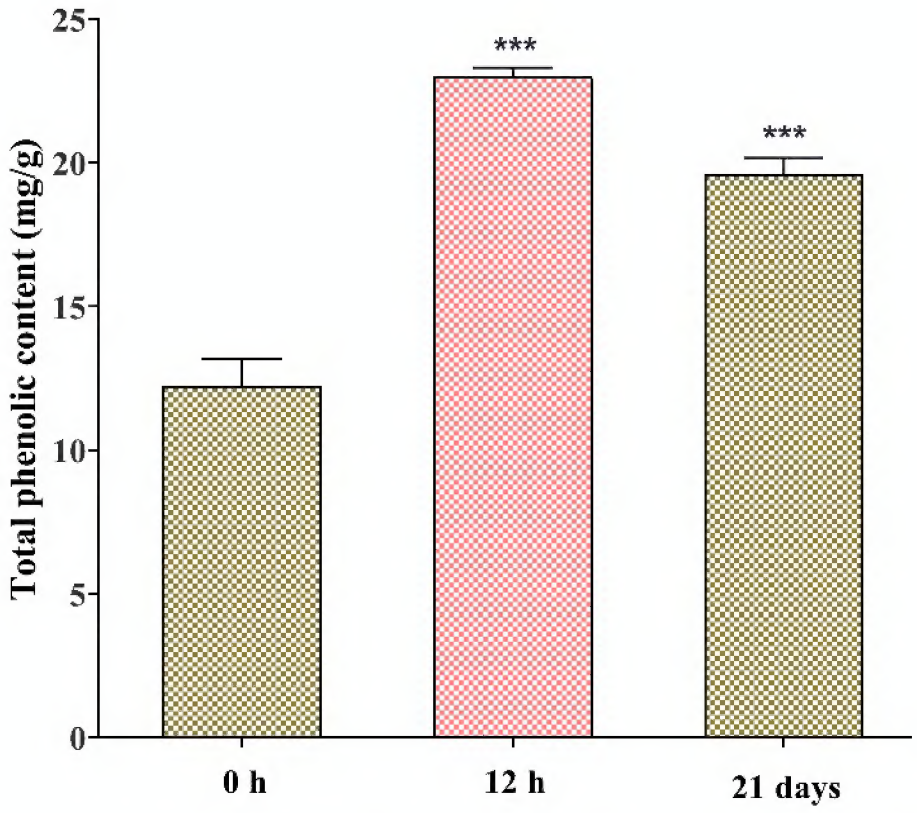

Figure 7. Quantification of phenolics in soymilk pre- (0 h) and post- (12 h and 21 days) *L. acidophilus* fermentation at 37 °C. Error bars represent the standard deviation (SD) from three independent experiments. Statistical significance; ns > 0.05,* p < 0.05, ** p < 0.005, *** p < 0.0005, compared to each other.

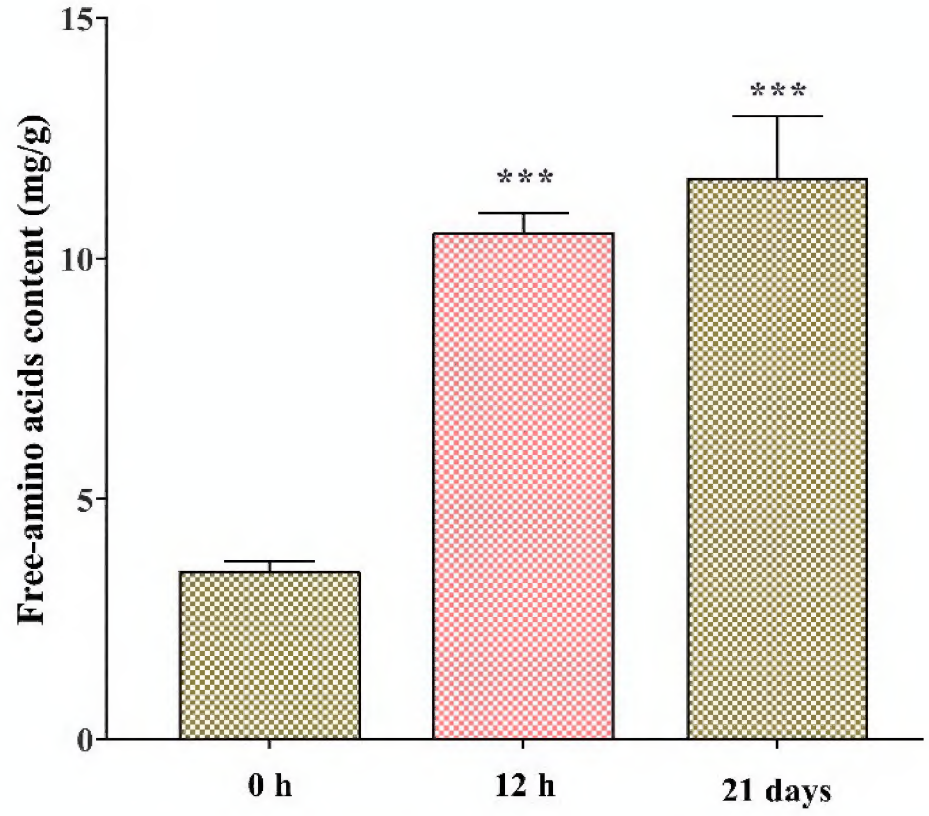

Figure 9. Quantification of amino acids in soymilk pre- (0 h) and post- (12 h and 21 days) *L. acidophilus* fermentation at 37 °C. Error bars represent the standard deviation (SD) from three independent experiments. Statistical significance; ns > 0.05,* p < 0.05, ** p < 0.005, *** p < 0.0005, compared to each other.

Antioxidant and anticancer potential of fermented soymilk

The antioxidative potential of the novel soymilk product was assessed by the DPPH• radical scavenging assay. The assay measured the decrease in the absorbance of DPPH free radicals due to the presence of fermented soymilk product. The fermented soymilk product showed a significant antioxidant activity against DPPH free radicals. The fermented soymilk product scavenged the DPPH• free radicals effectively, resulting in a lower absorbance. The antioxidative capability of the fermented soymilk product elevated with its concentration. The IC_{50} value (the concentration of fermented soymilk product that scavenged 50% of free radicals) was 55.51 µg/mL against DPPH (Fig. 10). The anticancer properties of fermented soymilk were tested by MTT assay against HCT-116 colorectal cancer cells. Fermented soymilk reduced the cell viability in a concentration-dependent way. Higher concentrations of fermented soymilk caused a notable decline in the viability of HCT-116 cells. Fermented soymilk had an IC_{50} value (67.13 µg/mL) against HCT-116 colorectal cancer cells (Fig. 11). The antioxidant and anticancer activities of fermented soymilk extract against DPPH and colorectal cancer cells (HCT-116) were influenced by the dose. This effect could be attributed to the elevated levels of phenols, flavonoids and free amino acids present in the fermented soymilk. Prior research indicates that the use of *S. thermophilus* 14,085 and *B. infantis* 14,603 in soymilk fermentation could inhibit the growth of Caco-2 and HT-29 cells. It's interesting to discover that the ethanol extract had a stronger antiproliferative effect (Lai et al. 2013). Another

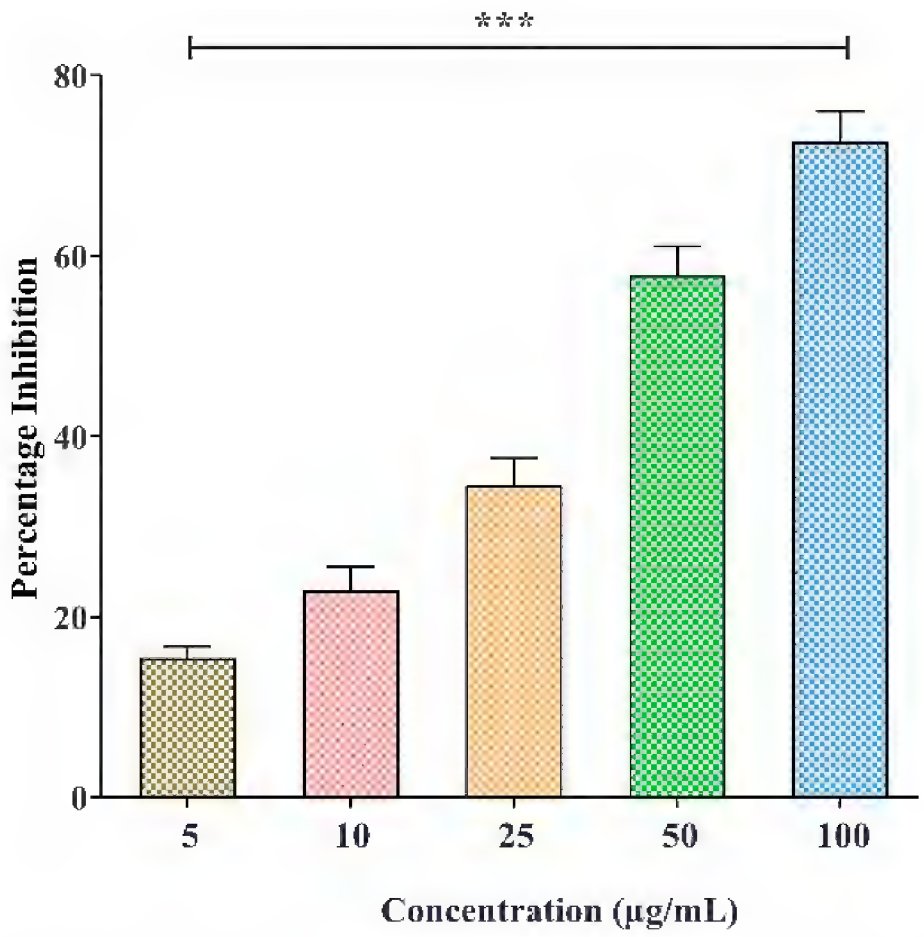

Figure 10. Impact of varying fermented soymilk extract concentrations on DPPH radical scavenging activity. Error bars represent the standard deviation (SD) from three independent experiments. Statistical significance; ns > 0.05,* p < 0.05, ** p < 0.005, *** p < 0.0005, compared to each other.

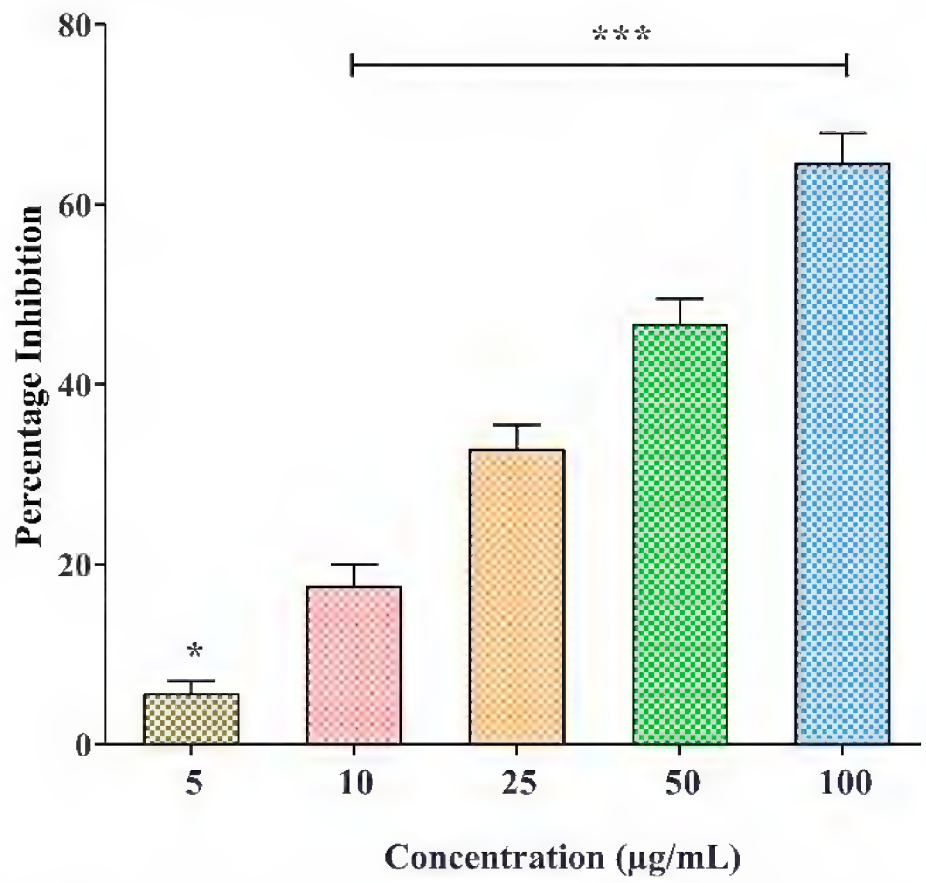

Figure 11. Impact of varying fermented soymilk extract concentrations on the proliferation of HCT-116. Error bars represent the standard deviation (SD) from three independent experiments. Statistical significance; ns > 0.05,* p < 0.05, ** p < 0.005, *** p < 0.0005, compared to each other.

study highlighted that fermented black soybeans exhibited notably superior antiproliferative properties against HT-29 and Caco-2 cells compared to unfermented soymilk, irrespective of whether the extracts were derived from water, 80% methanol, or 80% ethanol (Chen et al. 2013). Moreover, the soybean compound genistein was observed to influence the caspase-3 and p38 MAPK pathways at various transcriptional and protein levels. This modulation potentially explains its anticancer attributes against HT29 colon cancer cells (Shafiee et al. 2016). Furthermore, genistein demonstrated the capacity to inhibit S-phase kinase-associated protein 2 while elevating its subsequent targets, p21 and p27, within breast cancer cells, bolstering its potential as a tumor suppressor (Ye et al. 2018; Elkhalifa et al. 2023). In a related context, soybean seed powder subjected to fermentation with *L. plantarum* DGK-17 was found to instigate an antiproliferative response in human colon cancer HCT-116 cells, potentially mediated by the ROS-JNK signaling pathway (Khan and Kang 2017).

Conclusion

Our study highlights the development of fermented soymilk with probiotic *L. acidophilus*, a novel functional food with significant health benefits. The combination of soy's nutritional richness as well as probiotic benefits (*L. acidophilus*) not only improve sensory attributes of soymilk but also introduces a range of potential health benefits. The novel functional food revealed improved total phenolic, flavonoids and high in antioxidant activity. Additionally, the anticancer potential of novel functional food against the human colorectal cancer cells showed potential inhibition in dose dependent

manner indicating that it may potentially contribute for the management and prevention of a range of illnesses, including gastrointestinal disorders, immune system modulation and cancer. Meanwhile, as research in the field of functional foods continues to evolve, further investigations into the particular health benefits, bioavailability of bioactive compounds, and long-term effects of consuming fermented soymilk with *L. acidophilus* are warranted. Subsequently, this innovative approach to functional food development opens up possibilities for expanding the repertoire of plant-based, probiotic-rich products in the market, catering to the varied preferences and health-conscious choices of users. In essence, the fermented soymilk with probiotic *L. acidophilus* emerges as a promising candidate in the realm of functional foods, offering a delectable and health-enhancing option for those seeking nutritionally enriched alternatives.

Statements and declarations

Author contributions

Conceptualization, S.A.A. (Syed Amir Ashraf) and A.E. O.E., (Abd Elmoneim O Elkhalifa); methodology, E.A.S. (Eyad Al-Shammari), M.K. (Mohammed Kuddus), and M.P (Mitesh Patel); validation, A.M.A. (Amir Mahgoub Awadelkareem), M.S. (Manojkumar Sachidanandan) and Z. R. Azaz Ahmad Azad (Z R. A.A. Azad); formal analysis, M.P., S.A.A; investigation, S.A.A., M.P., M.K..; data curation, M.A. (Mohd Adnan), Z.R.A.A.A., E.A.S., A.M.A., and M.S.; writing—original draft preparation, A.E. O.E., A.M.A., M.P., M.A., and M.K.; writing—review and editing, S.A.A., M.A., M.S., and A. M. A.; software, Z.R.A.A.A., M.K., M.P., and A. E.O.E.; visualization, S.A.A., E.A.S., M.K., M.A., and A.M.A.; supervision, A.E.O.E., and S.A.A.; project administration, A.E.O.E. and S.A.A All authors have read and agreed to the published version of the manuscript.

Conflict of Interest

The authors declare that they have no known conflicting financial or relational interest that may have influenced the contents or methodology within this work.

Acknowledgements

This research has been funded by Scientific Research Deanship at University of Ha'il-Saudi Arabia through project number RG-21121.

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